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High molecular weight adiponectin activates AMPK and suppresses cytokine-induced NF-κB activation in vascular endothelial cells

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Abstract **Various isoforms of adiponectin circulate in the plasma. We purified high molecular weight (HMW) adiponectin from human plasma. HMW adiponectin was observed to activate AMP-activated protein kinase (AMPK), thereby increasing the phosphorylation of eNOS and NO production in endothelial cells. On the other hand, cells preincubated with HMW adiponectin had reduced TNFα-induced NF-κB activation. HMW adiponectin by itself was found to modestly activate NF-κB, which was significantly enhanced by inhibition of AMPK/eNOS activation. Thus, HMW adiponectin might have dual action, both pro and anti-inflammatory. An initial period of NF-κB activation by HMW adiponectin might be proinflammatory, but it could be counteracted by activation of AMPK/eNOS, which lead to a potential reduction in a second activation of NF-κB against inflammatory stimuli.**

Keywords: Adiponectin; AMPK; NO; NF-κB; Endothelial cells

1. Introduction

Adiponectin is an adipocyte-specific protein that enhances insulin sensitivity and promotes lipid metabolism [1,2]. Adiponectin circulates in plasma as three forms: a trimer (low molecular weight: LMW), a hexamer (trimer–dimer) of medium molecular weight (MMW), and a larger multimeric high molecular weight (HMW) form [3–6]. A proteolytic cleavage product of adiponectin, known as globular adiponectin (gAd), also appears to circulate in human plasma [7]. The biologic activity of each isoform has not been firmly established, but it appears that HMW adiponectin has a beneficial role in humans and rodents with regard to prevention against the development of atherogenesis. Experimental and clinical data suggest that the oligomeric complex distribution of adiponectin is essential for its anti-diabetic and anti-atherogenic activity [8,9]. Thus, we obtained HMW adiponectin from human plasma by utilizing its affinity for gelatin-Cellulofine and investigated its effect on vascular endothelial cells. In the present study, we evaluate the potency of HMW adiponectin on AMP-activated protein kinase (AMPK) activation and nuclear factor (NF)-κB activation.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA, USA) and cultured in EBM medium supplemented with 5% fetal calf serum in the standard fashion. The cells in this experiment were used within 3–4 passages and examined to ensure that they demonstrated the specific characteristics of endothelial cells. Mouse SVEC4 cells (axillary lymph node, vascular endothelial; SV40 transformed) were also cultured in DMEM containing 10% fetal calf serum and observed to demonstrate the typical cobblestone morphological appearance of endothelial cells.

2.2. Western blot analysis

HUVEC were lysed in buffer comprising 10 mmol/L Tris (pH 7.4), 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L NaF, 20 mmol/L Na_4P2O_7, 2 mmol/L Na_3VO_4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X 100, 10% glycerol, 10 μg/mL leupeptin, 60 μg/mL aprotinin, and 1 mmol/L phenylmethanesulfonyl fluoride. HUVEC lysates were resolved on SDS–PAGE according to standard protocols. After being transferred to membranes, the samples were immunoblotted with primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase. Bands were revealed using an enzyme-linked chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ), and density was quantified with a LumiVision Analyzer (Aisin, Kariya, Japan). The primary antibodies used were as follows: anti-phosphorylated AMPKα (Thr-172), anti-AMPKα, anti-phosphorylated acetyl-CoA carboxylase (ACC) (Ser-79), anti-ACC, anti-phosphorylated eNOS (Ser-1177) (Cell Signaling Technology, Beverly, MA), and anti-eNOS monoclonal antibody (BD Biosciences, San Jose, CA).

2.3. Microarray analysis

Microarray analysis of cDNA from the RNA prepared from untreated HUVEC or HUVEC treated with HMW adiponectin for 8 h was performed using Affymetrix GeneChip Expression Analysis (containing oligonucleotide probe sets for approximately 8500 human genes).

2.4. NF-κB activation

To study NF-κB activation, SVEC4 cells were stably transfected with a *cis*-reporter plasmid containing the luciferase reporter gene linked to five repeats of NF-κB binding sites (pNFκB-Luc: Stratagene,

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La Jolla, CA, USA), as previously described [10]. For this, the pNFκB-Luc plasmid was transfected together with a pSV2neo helper plasmid (Clontech, Palo Alto, CA, USA) into SVEC4 cells using a FuGEN 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany). The cells were then cultured in the presence of G418 (Clontech) at a concentration of 500 μg/mL and the medium was replaced every 2–3 days. Approximately 3 weeks after transfection, G418-resistant clones were isolated using a cloning cylinder and analyzed individually for expression of luciferase activity. Several clones were also selected for analysis of NF-κB activation. Luciferase activity was measured using a luciferase assay kit (Stratagene).

In order to study NF-κB activation in HUVEC, cells were transfected with the pNFκB-Luc plasmid at 48 h prior to the experiments. Constitutively active pCMV-*Renilla* vectors were used for normalization of transfection efficiency. The two luciferase signals were assessed with a dual luciferase assay kit.

2.5. Real-time PCR

For quantitative measurement of mRNA, 2 μg total RNA was treated with DNase I for 15 min and subsequently used for cDNA synthesis. The PCR reactions using cDNA were performed in a LineGene system (BioFlux, Tokyo, Japan) under the following conditions: 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s.

2.6. siRNA transfection

The day before transfection, plates were inoculated with an appropriate number of HUVEC in serum-containing medium to ensure 50–70% confluence the following day. LKB1 siRNA or CaMKKα siRNA (Santa Cruz Biotechnology Inc.) mixed with siLentFect (Bio-Rad) was added to the cells at a concentration of 10 nM. Forty-eight hours after transfection, AMPK activation induced by HMW adiponectin was assessed.

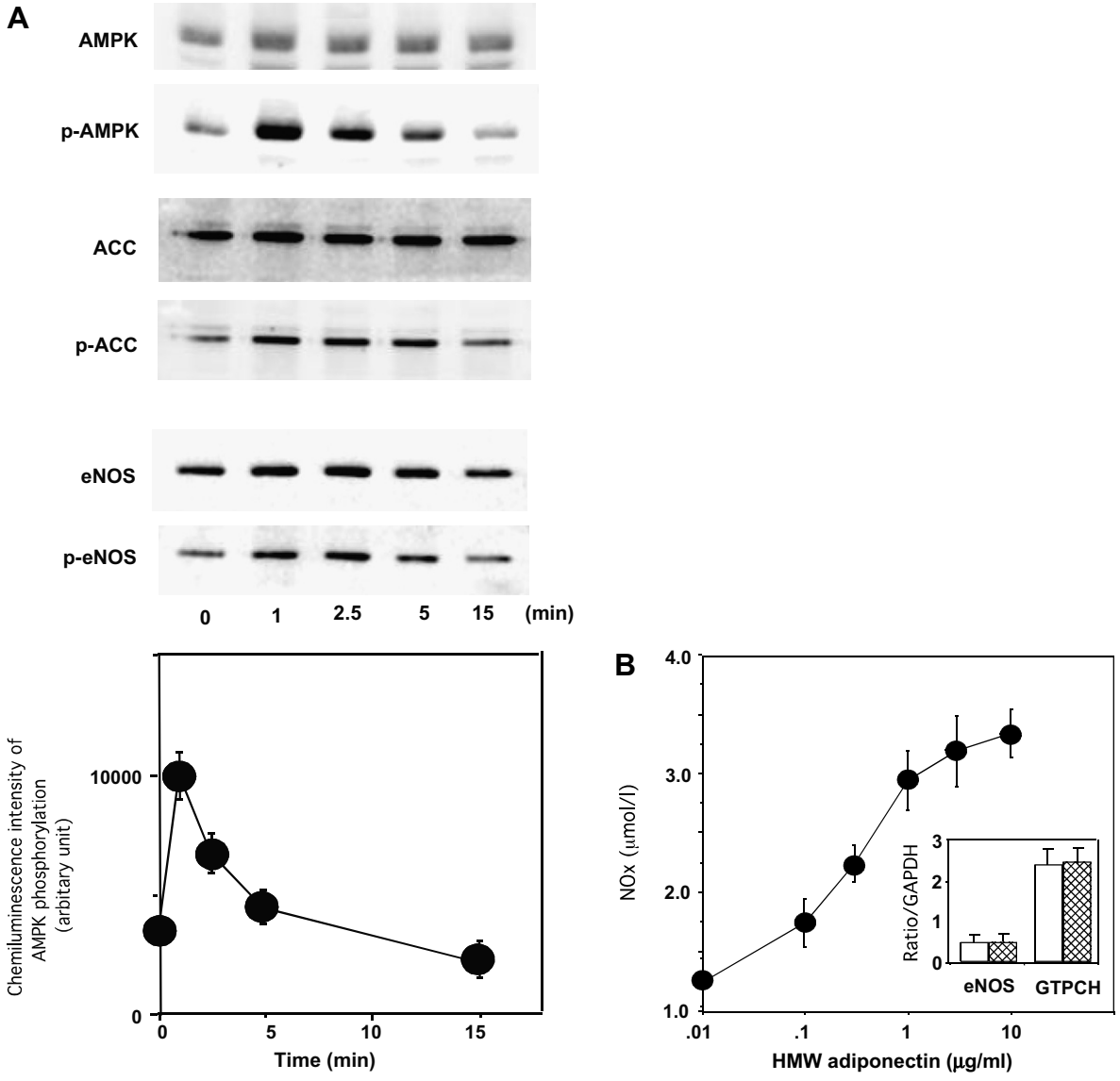

Fig. 1. HMW adiponectin activates AMPK in vascular endothelial cells. (A) HUVEC were treated with 10 μg/mL HMW adiponectin for the indicated time periods before lysis, after which cell lysates were probed with antibodies specific for AMPK, ACC, or eNOS, or their phosphorylated. The chemiluminescence intensity for HMW adiponectin- and gAd-induced phosphorylated AMPK from three independent experiments was quantified. (B) HMW adiponectin dose-dependently increase NO production in HUVEC. Inset: mRNA levels for eNOS and GTPCH. mRNA levels were expressed as the ratio to the GAPDH, white bar: control, hatched bars: HMW adiponectin (10 μg/mL) for 6 h.

Materials. HMW adiponectin was purified from human plasma as previously described [3], which is endotoxin free according to the limulus test. HMW adiponectin primarily exists as a 18 mer adiponectin. siRNAs for LKB1, CaMKKα , and eNOS were obtained from Santa Cruz Biotechnology Inc., BAY 11-7082 was obtained from Biomol (Plymouth Meeting, PA).

2.7. Statistical analysis

The results are expressed as mean values ± S.D. ANOVA and Fisher's least significant difference test were used for multigroup comparisons, with a P value of less than 0.05 considered significant.

3. Results

3.1. Adiponectin activates AMPK in HUVEC

Treatment of HUVEC with HMW adiponectin (10 μg/mL) resulted in time-dependent activation of AMPK, as monitored by phosphorylation of AMPK and its down-stream target, ACC (Fig. 1A). This may have resulted in eNOS phosphorylation and activation. Indeed, Ser1177 phosphorylation of eNOS was observed to peak at 2.5 min. Fig. 1A (lower panel) shows the time course of changes in the chemiluminescence intensity of AMPK phosphorylation by HMW adiponectin.

We confirmed eNOS activation by HMW adiponectin by measuring the concentration of bioactive NO in the cellular supernatant (NO_x, as measured by NO^{2-} and NO^{3-} levels). Incubation of HUVEC with HMW adiponectin dose-dependently increased NO_x concentrations in the culture medium (Fig. 1B). Examination of the time course showed a substantial increase in NO production over the course of 1 h, after which only a modest increase in NO production was observed (data not shown). The inset in Fig. 1B shows that HWM adiponectin did not change mRNA levels of eNOS and GTP cyclohydrolase (rate limiting enzyme for de novo synthesis of tetrahydrobiopterin).

3.2. CaMKKβ mediates AMPK activation upon adiponectin stimulation

AMPK is controlled by upstream kinases, which have been identified as LKB1 or Ca^{2+}/calmodulin-dependent protein kinase β (CaMKKβ) [11]. Both LKB1 and CaMKKβ are expressed in HUVEC (data not shown). In order to assess whether CaMKKβ or LKB1 might act as an AMPK kinase (AMPKK) in adiponectin-stimulated cells, we used an siRNA approach to knock down the expression of LKB1 or CaMKKβ. Compared with results following transfection using control siRNA, HMW adiponectin-induced AMPK activation was significantly reduced in cells treated with CaMKK si RNA, but not in cells treated with LKB1 siRNA (Fig. 2A). To further address this question, we performed experiments with STO-609, a relatively selective inhibitor of CaMKK

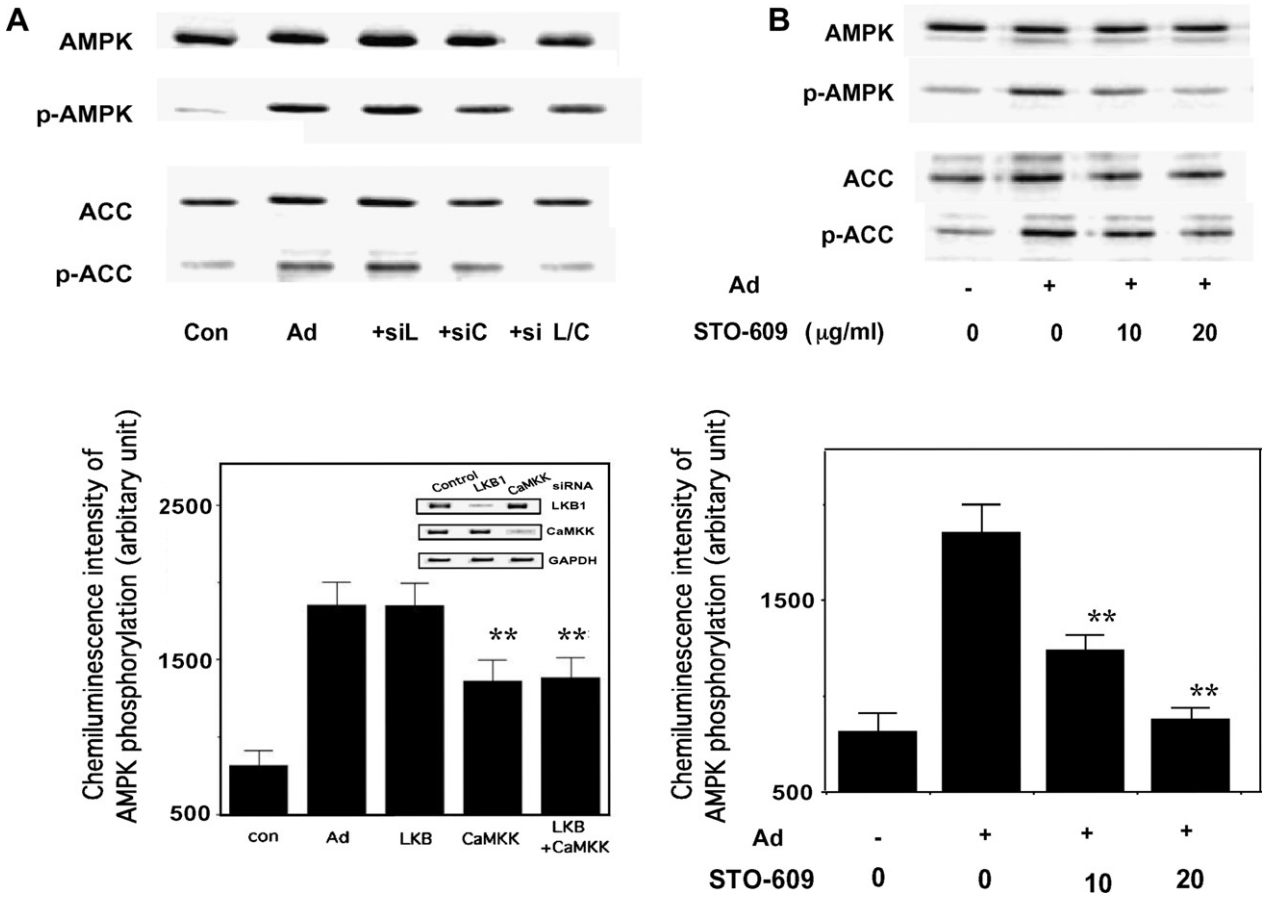

Fig. 2. (A) HMW adiponectin (Ad) activates AMPK, which was significantly attenuated in HUVEC transfected with CaMKKβ siRNA (siC: 10 nM) but not with LKB1 siRNA (siL: 10 nM). Inset (lower figure): 48 h after cells were transfected with control, LKB1, or CaMKKβ siRNA, the mRNA levels of LKB1 or CaMKKβ were determined. (B) HMW adiponectin (Ad)-induced AMPK phosphorylation was inhibited by the inhibitor of CaMKKβ, STO-609 in HUVEC. Results represent the means ± S.D. ($n = 4$). $^{**}P < 0.01$ vs. AMPK activity by Ad.

[12]. STO-609 also caused a dose-dependent reduction in HMW adiponectin-induced AMPK phosphorylation (Fig. 2B).

3.3. Adionectin activates NF-κB

We examined whether HMW adiponectin induces NF-κB activity in cells. HMW adiponectin was observed to dose-dependently activate NF-κB-mediated gene transcription (Fig. 3A). However, NF-κB-dependent transactivation by HMW adiponectin at concentration of 10 μg/mL was much smaller than gAd, TNFα, LPS, and IL-1α (Fig. 3B) compared to untreated cells. This activation of NF-κB was significantly enhanced by inhibition of NO production with N^{ω}-methyl-L-arginine (L-NMMA), or AMPK inhibitor compound C (Fig. 3C).

3.4. Effect of adiponectin on B TNFα-Induced NF-κB activation

We then examined pre-treatment of cells with HMW adiponectin on TNFα-induced NF-κB-mediated gene transcription. In cells pre-treated with HMW adiponectin, TNFα-induced NF-κB activity was dose-dependently inhibited with greater inhibition observed with longer periods of incubation (Fig. 4A). Incubation for 16 h with TNFα substantially induced VCAM-1 gene expression in HUVEC. The degree of induction of VCAM-1 mRNA by TNFα was modulated by length of exposure to HMW adiponectin prior to TNFα (data not shown).

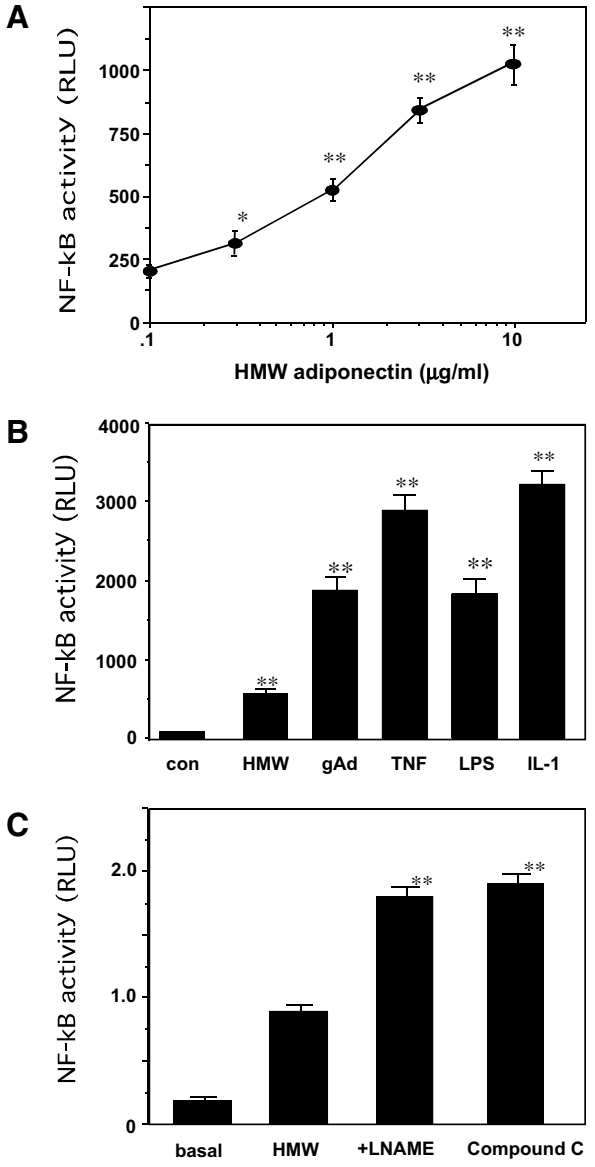

Fig. 3. The effects of HMW adiponectin and globular adiponectin on NF-κB-dependent transcriptional activity. (A) SVEC4 cells (transfected with pNFκB-Luc) were left untreated or were treated with various concentrations (1–10 μg/mL) of HMW adiponectin. After 2 h, cells were lysed, and luciferase activity was measured. (B) HMW adiponectin activates NF-κB in SVEC4 cells, which was compared with that induced by gAd (10 μg/mL), TNFα (10 ng/mL), LPS (1 μg/mL), or IL-1α (10 ng/mL). (C) HMW adiponectin-induced activation of NF-κB was significantly enhanced in HUVEC treated with L-NMMA (1 mM), or compound C (10 μM). NF-κB activity was expressed as a ratio of two signals co-transfected firefly and *Renilla* luciferases. Results represent the means ± S.D. ($n = 4$). $^{**}P < 0.01$ vs. NF-κB activity by HMW adiponectin.

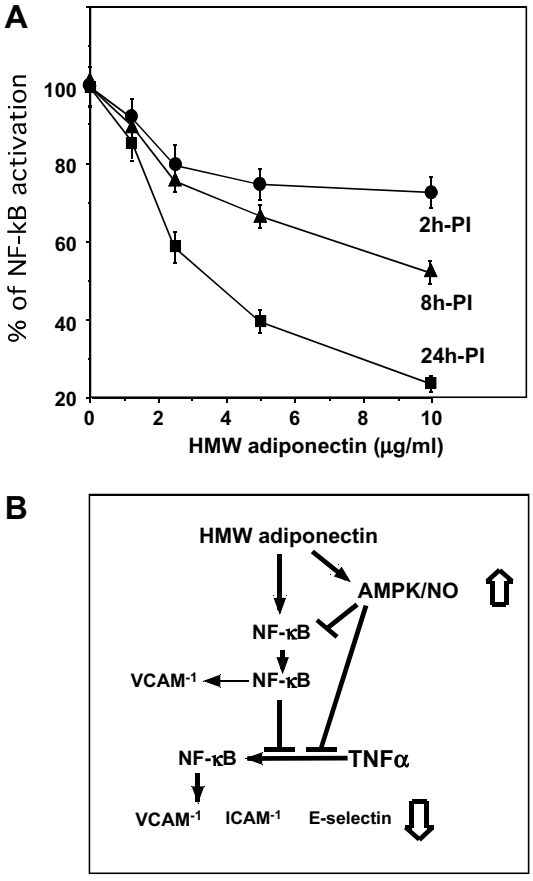

Fig. 4. (A) The effect of HMW-adiponectin pretreatment on TNFα-induced NF-κB-dependent transcriptional activity. SVEC4 cells (transfected with pNFκB-Luc) were treated with various concentrations of adiponectin for 2, 8, or 24 h and then treated with TNFα for 2 h. Then, cells were lysed, and luciferase activities were measured. 2 h-PI (closed circles), 8 h-PI (closed triangles), and 24 h-PI (closed squares): preincubation for 2, 8, and 24 h. Data are means ± S.D. of triplicate observations. (B) Schematic diagram illustrating the proposed effect of HMW adiponectin leading to suppression of TNFα-stimulated responses in endothelial cells.

Table 1
DNA microarray analysis and real-time PCR

	DNA macroarray analysis			Real-time PCR	
	Base (*B*) signal	Exp (*E*) signal	*E*/*B* ratio	*E*/*B* ratio (±S.D.)	*E*/*B* ratio + BAY11-7082
HMW-adiponectin					
ICAM-1	64.1	102.5	1.6	1.85 ± 0.12	0.46 ± 0.06**
E-selectin	7.4	16.2	2.2	2.65 ± 0.29	1.18 ± 0.11**
VCAM-1	6.7	13.4	2.0	2.45 ± 0.09	0.76 ± 0.08**

** $P < 0.01$ vs. E/B ratio in the absence of BAY11-7082.

3.5. Microarray and real-time PCR analysis (Table 1)

Microarray analysis of cDNA from RNA prepared from untreated HUVEC or HUVEC treated with HMW adiponectin (10 μg/mL) for 8 h was performed using Affymetrix GeneChip Expression Analysis (containing oligonucleotide probe sets for approximately 8500 human genes). We found that HMW adiponectin increased the expression of ICAM-1 (1.6-fold), E-selectin (2.2-fold), and VCAM-1 (2.0-fold). We confirmed these increases in mRNA expression by real-time PCR, with significant inhibition of expression observed using the NF-κB inhibitor BAY11-7082 [13], suggesting that HMW adiponectin activates NF-κB.

4. Discussion

In the present study, we demonstrate that HMW adiponectin activates AMPK by promoting AMPK phosphorylation. Although we did not perform a strict kinase assay for AMPK, we are certain that HMW adiponectin activates AMPK since the extent of AMPK phosphorylation at Thr-172 strongly reflects its activity [14], and since phosphorylation of the AMPK consensus substrate, ACC, at Ser-79 was also observed. Active AMPK in turn activates eNOS through phosphorylation of Ser-1177, which is thought to be the consensus phosphorylation site of AMPK [15], thereby inducing NO production in HUVEC. Thus, AMPK activation by HMW adiponectin appears to play a protective role against endothelial inflammation and atherogenesis. Our study examines CaMKKβ expression and its functional significance with regard to AMPK activation by adiponectin. Two separate lines of evidence, pharmacological inhibition of CaMKKβ by STO-609 [12] and downregulation of CaMKKβ with specific targeted siRNA, suggest that CaMKKβ mediates AMPK activation by adiponectin.

Using microarray analysis we found that HMW adiponectin increased the expression of ICAM (1.6-fold), E-selectin (2.2-fold), and VCAM-1 (2.0-fold). This induction of gene expression is dependent on NF-κB activation, and is significantly inhibited by the NF-κB inhibitor BAY11-7082 [13], suggesting that HMW adiponectin activates NF-κB. We therefore further examined whether HMW adiponectin really induces NF-κB activity in endothelial cells. HMW adiponectin was observed to dose-dependently activate NF-κB-mediated gene transcription. These results suggest that HMW adiponectin possess pro-inflammatory properties. However, this increase in NF-κB activation was much weaker than the same concentration of gAd. gAd, a proteolytic cleavage product of adiponectin, appears to activate vascular endothelial cells [16]. gAd has been shown to account for less than 1% of the total adiponectin in blood [17]. Therefore, the biological meaning of adiponectin should be evaluated in terms of the physiological distribution of its various isoforms, including HMW adiponectin. HMW adiponectin produced a lesser increase in NF-κB activity than TNFα, LPS, or IL-1α. The modest nature of this increase in NF-κB activation may be due, at least in part, to simultaneous NO production following activation of AMPK/eNOS by HMW adiponectin. Indeed, when AMPK was inhibited by compound C or NO production was inhibited by the treatment with L-NMMA, NF-κB activation significantly increased.

We demonstrated that pretreatment with HMW adiponectin dose-dependently decreased NF-κB activation in accordance with length of pretreatment. This occurred following an initial period of activation of NF-κB by adiponectin. Reduced responsiveness or desensitization to TNFα might occur after an initial activation during pretreatment. AMPK activation and/or NO production likely attenuates activation of NF-κB permitting modest VCAM expression but protecting against inflammatory mediators (Fig. 4B). In fact, a recent report shows positive associaton of adiponectin with soluble vascular cell adhesion molecule (sVCAM) levels but negatively with hs-CRP in patients with vascular disease or dyslipidemia [18].

We previously showed that gAd potently activates NF-κB in vascular endothelial cells, which in turn induces expression of proinflammatory and adhesion molecule genes [16]. Although Waki et al. have shown cleavage of adiponectin by leukocyte elastase secreted from activated monocytes and/or neutrophils [6], it seems that minimal amounts of gAd are found even at sites of inflammation. Here, HMW adiponectin was found to modestly activate NF-κB, and appeared to require a shorter preincubation period to inhibit TNFα-induced NF-κB activation, compared with globular adiponectin. Both gAd and HMW adiponectin activate AMPK and stimulate NO production. Thus, both gAd and HMW adiponectin appear to interact with the same receptors on vascular endothelial cells to activate AMPK and NF-κB. The manner by which gAd and HMW adiponectin bind to the receptors, as well as the post-binding events, likely differs. As we previously showed, antibodies against the extracellular domains of AdR1 strongly activate NF-κB [19]. Therefore, how adiponectin stimulates AdR1 should be clarified to understand the mechanism of action of adiponectin. Under normal conditions, high concentrations of HWM adiponectin are present in vessels. This indicates that adiponectin might be protective against inflammatory stimuli.

In conclusion, we suggest that HMW adiponectin might have dual action, both pro and anti-inflammatory on vascular endothelial cells. An initial period of NF-κB activation by HMW adiponectin might be proinflammatory, but it could lead to a potential reduction in a second activation of NF-κB in response to inflammatory stimuli.

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